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FEATURE

Environmentally-benign reaction chemistry using critical fluids — analytical implications

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Reactions conducted in pressurized fluid media (supercritical fluids) have several distinct advantages in terms of speed, selectivity, and product quality. Using carbon dioxide under high pressure, GRAS (Generally Regarded as Safe) solvents or hot pressurized water also allows compliance with environmental and worker safety regulations. Reactions in such media can be conveniently combined with extraction or fractionation steps, and used in both processing and analytical chemistry applications. This paper describes applications in lipid analysis; the implications for lipid processing were examined in an earlier paper in the September 2001 issue of Lipid Technology journal.

Introduction

In recent years, pressurized gases or fluids, called supercritical and subcritical fluids, have been employed as alternatives to potentially toxic organic solvents in unit processes such as extraction, fractionation and reactions. The reason is that the increasing environmental regulation of both industrial processes and laboratory environments has given rise to the concept of 'green' technologies which have an environmentally-benign effect. We described developments in processing applications for supercritical and subcritical fluids in the September 2001 issue of *Lipid Technology*

(pages 109–113) and in this paper will discuss how reactions in such media can be applied in analytical chemistry for sample preparation and derivatization.

As we discussed in the earlier paper, there are numerous advantages to using a pressurized gas or fluid at high density for synthetic purposes. These include: improvement in mass transfer of reactants and products; control of reactants and product solubility; the use of lower reaction temperatures; the control of the reaction rate by manipulating pressure; and alteration of the final product distribution or characteristics.

<u>AOCS method</u>	<u>SFE/SFR method</u>
<ul style="list-style-type: none"> • Alcoholic saponification • Evaporate alcohol; dilute with H₂O; neutralize; cool • Extract with pet. ether • Filter the combined extracts • Wash filter paper • Evaporate pet. ether • Dry fatty acids in oven • Cool in desiccator to ambient temperature and weigh • Repeat until constant weight 	<ul style="list-style-type: none"> • Mix with Hydromatrix • Freeze dry • SFE/SFR • GC/FAME analysis
Total time 5–8 hours (solvent: 575 ml)	Total time 3 hours (solvent: 1.8 ml)
	<u>SFC method</u>
	<ul style="list-style-type: none"> • Neutralize (if necessary) • Extract with diethyl ether • Add internal standard • SFC analysis
	Total time 45 minutes (solvent: 8 ml)

Figure 1. A comparison of the steps involved in three methods for determining the free fatty acid content of industrial soapstocks: (i) American Oil Chemists' Society Method G3-53; (ii) supercritical fluid extraction combined with supercritical fluid reaction (SFE/SFR); and (iii) supercritical fluid chromatography (SFC). GC = gas chromatography; FAME = fatty acid methyl esters.

The relatively high solubilities of lipids in supercritical carbon dioxide (SC-CO₂), and the miscibility of oils and fats in high-temperature pressurized water, mean that these two media can be good solvents in which to conduct lipid chemistry. They also meet the 'green' criterion and can be discharged into the environment with a relative benign effect.

Fat determination in foods using critical fluids

Developments in our processing research using supercritical and near-critical solvents have provided opportunities to exploit the technology for analytical purposes. From our early success in performing selected esterifications to fatty acid methyl esters (FAME) formation in SC-CO₂ through enzymatic catalysis, we were able to transfer the techniques into the analytical area, particularly for the analysis of fat levels in various foods.

A primary emphasis was placed in the analysis of the fat content of meats for the US Food Safety and Inspection Service (FSIS) according to the guidelines developed for fat definition and quantification under the Nutritional Labeling and Education Labeling Act (NLEA). Here fat content was determined by

performing a total fatty acid analysis (as the FAME derivatives) on a fat sample obtained by supercritical fluid extraction (SFE). A logical pattern of investigations was developed that explored effects of the food matrix (for example, levels of fat and moisture, and type of lipid) on the corresponding FAME profile, different sample types (meats, oilseeds, grain/cereal products) and the best enzyme (lipase) for derivative formation.

Using extraction/reaction conditions of 12.2 MPa and 50°C with Novozyme 435, very reproducible and quantitative FAME distributions could be obtained on three different types of dehydrated meat matrices of varying fat content (15–40% by weight.). Good agreement was achieved between fat contents determined by enzymatic formation of FAME from SFE and by FAME derived from chemical derivatization and solvent extraction done at an independent laboratory. Methyl ester formation from model lipid compounds besides triacylglycerols (for example, phospholipids and sterol esters) indicated complete extraction and conversion of these moieties using extraction with SC-CO₂ followed by lipolysis over Novozyme 435.

Considerable effort in our laboratory, as well as in others, culminated in Official American Oil Chemists' Society (AOCS) Method Am3-96, 'Oil in Oilseeds: Supercritical Fluid Extraction Method' which is now in routine use for determining the oil content of vegetable seeds. Additional cross-comparison studies between the NLEA method and classic gravimetric fat assays have been undertaken. These have resulted in an appreciation of the factors which account for the discrepancy between the two methods and their definition of fat.

The studies have included the use of automated SFE instrumentation and the translation of the FAME-based method into an entirely automated procedure that involves fat extraction, derivatization (during extraction) to form FAME, and robotic transfer of the derivatized sample for overnight analysis by gas chromatography (GC). These results have also compared favourably with fat levels for a variety of foodstuffs determined with the manual NLEA methods that use SFE or organic solvent.

FFA in soapstocks

An extraordinary example of application of the SFE/SFR (supercritical fluid reaction) technique was developed in our laboratory as an alternative to the standard AOCS method for determining the free fatty acid content of industrial soapstocks. The standard method uses large quantities of solvent and is time-consuming. In this case, a company was concerned with the cost and time incurred while analysing the fatty acid content of soapstocks delivered by tanker trucks.

As shown in Figure 1, application of the SFE/SFR method to a soapstock sample partially dehydrated by freeze-drying allows the GC/FAME analysis to be performed in under 3 hours using less than 2 ml of solvent. This is in contrast to AOCS Method G3-53 which requires many manual steps, 5-8 hours to perform depending on the analyst, and 575 ml of organic solvent. Interestingly, a qualitative SFC method can be used to detect gross differences in the fatty acid content of various soapstock samples in under 45 minutes as noted in Figure 1.

Fat-soluble vitamins

Researchers at Lund University in Sweden have recently used an integrated enzyme-initiated reaction to hydrolyse fat-soluble vitamins during the SFE of vitamins from a variety of food matrices. Using Novozyme 435 at 60°C and 25.9 MPa (SC-CO₂ density of 0.8 g/ml), and 5% by volume of ethanol, they have successfully extracted food matrices containing vitamins A and E.

They found that vitamin A could be readily hydrolysed under SC-CO₂ conditions to retinol which could be quantified by HPLC using ultraviolet and/or fluorescence detection. However, vitamin E was sterically inhibited from entering the active site of the enzyme, and could not be hydrolysed to any appreciable extent. Nevertheless, vitamin E analyses were performed successfully on many of the chosen food samples that had a large quantity of naturally-occurring α -tocopherol: milk powder, infant formula, liver paste, and minced meats.

Recoveries of retinol from the above matrices were from 79-119%, averaging 100.8%, when compared to a collaborative study done in the European Union using only SFE for the extraction of vitamins. The enzyme

Table 1. A comparison of the methanolysis activities of different enzymes in supercritical carbon dioxide toward three lipid substrates: a commercial shortening; cholesteryl stearate (C₁₈CE); and phosphatidylcholine (PC).

Enzyme source	Activity (%)		
	Shortening	C ₁₈ CE	PC
Novozyme 435	100	98	99
Lipase G	90	100	48
Chirazyme L-1	100	98	90
Lipozyme IM	99	96	60

bed (Novozyme 435) could be used up to four times consecutively without a noticeable loss in hydrolytic efficiency.

Determining enzyme activity

The SFE/SFR technique can be 'inverted' to permit the evaluation of enzymatic activity under supercritical conditions, and hence the efficiency of different enzymes for a specific task. This can be done quite conveniently and rapidly using automated analytical SFE instrumentation in a combinatorial mode.

The results on the most promising enzyme candidates for conducting methanolysis at 17.2 MPa and 50°C for 80 minutes are shown in Table 1. Three lipid substrates were examined: a commercial shortening, cholesteryl stearate, and phosphatidylcholine. Note that Novozyme 435 assures methanolysis of all of the above lipid moieties under the stated conditions, while Lipase G, Lipozyme IM, and Chirazyme L-1 were slightly inferior and substrate dependent. Also note that eight other lipases in this study failed to show sufficient catalytic activity under the above conditions to warrant further use.

The results are shown in Table 2 for the enzymatic hydrolysis of vitamin A (retinyl palmitate) at 25.3 MPa and 60°C for 25 minutes by three lipases: *Candida antarctica* B,

Table 2. A comparison of the activities of enzymes from three different sources to hydrolysis of retinyl palmitate in supercritical carbon dioxide.

Enzyme source	Activity (%)
<i>Candida antarctica</i>	79
<i>Rhizomucor miehei</i>	57
<i>Pseudomonas cepacia</i>	41

Pseudomonas cepacia, and *Rhizomucormiehei*. At a water activity level of 0.43, it was apparent that the lipase derived from *Candida antarctica* is preferred for hydrolysis of Vitamin A in the presence of SC-CO₂. Three other lipases and one esterase in the above evaluation did not show sufficient hydrolytic activity to warrant further investigation. Readers should note that the activity levels and reaction efficiencies may change with the solvent media, the water activity, and the type of conversion being attempted with a specific enzyme.

Conclusions

In our earlier paper in *Lipid Technology* we concluded that effective reaction chemistry and engineering of lipids can be accomplished in a variety of critical fluid media under environmentally-benign conditions. We stated our hope that some of the described technology would be considered as a viable option to assure compliance with environmental and worker safety legislation. This present paper has shown how developments in our processing research using supercritical and near-critical solvents have provided opportunities to exploit the technology for analytical purposes.

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